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Whole-Genome Analyses of Speciation Events in Pathogenic Brucellae†

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Despite their high DNA identity and a proposal to group classical *Brucella* species as biovars of *Brucella melitensis*, the commonly recognized *Brucella* species can be distinguished by distinct biochemical and fatty acid characters, as well as by a marked host range (e.g., *Brucella suis* for swine, *B. melitensis* for sheep and goats, and *Brucella abortus* for cattle). Here we present the genome of *B. abortus* 2308, the virulent prototype biovar 1 strain, and its comparison to the two other human pathogenic *Brucella* species and to *B. abortus* field isolate 9-941. The global distribution of pseudogenes, deletions, and insertions supports previous indications that *B. abortus* and *B. melitensis* share a common ancestor that diverged from *B. suis*. With the exception of a dozen genes, the genetic complements of both *B. abortus* strains are identical, whereas the three species differ in gene content and pseudogenes. The pattern of species-specific gene inactivations affecting transcriptional regulators and outer membrane proteins suggests that these inactivations may play an important role in the establishment of host specificity and may have been a primary driver of speciation in the genus *Brucella*. Despite being nonmotile, the brucellae contain flagellum gene clusters and display species-specific flagellar gene inactivations, which lead to the putative generation of different versions of flagellum-derived structures and may contribute to differences in host specificity and virulence. Metabolic changes such as the lack of complete metabolic pathways for the synthesis of numerous compounds (e.g., glycogen, biotin, NAD, and choline) are consistent with adaptation of brucellae to an intracellular life-style.

Brucellosis is a major infectious disease affecting animals and humans (11). Several *Brucella* species, such as *Brucella abortus*, *Brucella melitensis*, and *Brucella suis*, have been isolated from a variety of animals. Of these, *B. abortus*, the causative agent of bovine brucellosis, is the most widespread (11). All three *Brucella* species cause a severe human disease characterized in its acute phase by undulant fever and in its chronic phase by localization of the pathogen and damage of different organs (11, 38). If localized in the brain or the heart, this can result in fatal meningitis or fatal endocarditis, respectively. *Brucella* infection is treated with a combination of antibiotics; however, in its chronic phase, eradication is difficult since *Brucella* spp. are intracellular pathogens, which puts them out of reach of humoral immunity and several antibiotics (25, 38). The lack of a safe and efficacious human vaccine underscores the importance of understanding the biology of brucellosis to develop human vaccines and effective therapeutic agents. Furthermore, all three *Brucella* species are listed as potential bioweapons by the Centers for Disease Control and Prevention (22, 26). This is due to the highly infectious nature of all three species, the fact that they can be readily aerosolized, and the fact that an outbreak would be difficult to detect because the initial symptoms are easily confused with those of influenza.

Brucella spp. belong to a monophyletic branch of the alpha 2 subgroup of proteobacteria, whose members share the ability to engage in intimate or sometimes intracellular associations with eukaryotic cells (48). However, little is known regarding the biological basis of *Brucella* sp. host specificity or about the mechanisms involved in the intracellular multiplication and persistence of members of this group. The availability of the *B. abortus* strain 2308 sequence reported here, together with the sequences of *B. suis*, *B. melitensis*, and *B. abortus* strain 9-941 (12, 18, 35), enabled us to perform a comprehensive examination and comparison of the gene composition, mutations, structural arrangement, and other characteristics of these genomes. Detailed comparisons have confirmed the *B. abortus* deletions identified with *B. melitensis*-derived microarrays (37) and have uncovered a large number of additional differences in *B. abortus*, as well as in *B. melitensis* and *B. suis*. Overall, our analyses confirm the striking similarities that exist among the three species and reveal a number of important, though subtle, features that may be important in host specificity and the adaptation of these organisms to an intracellular life-style.

MATERIALS AND METHODS

Genome sequencing and annotation. Genomic DNA was isolated from *B. abortus* strain 2308, a standard laboratory biovar I strain virulent for humans, cattle, and certain other domestic animals (40). Whole-genome sequencing was

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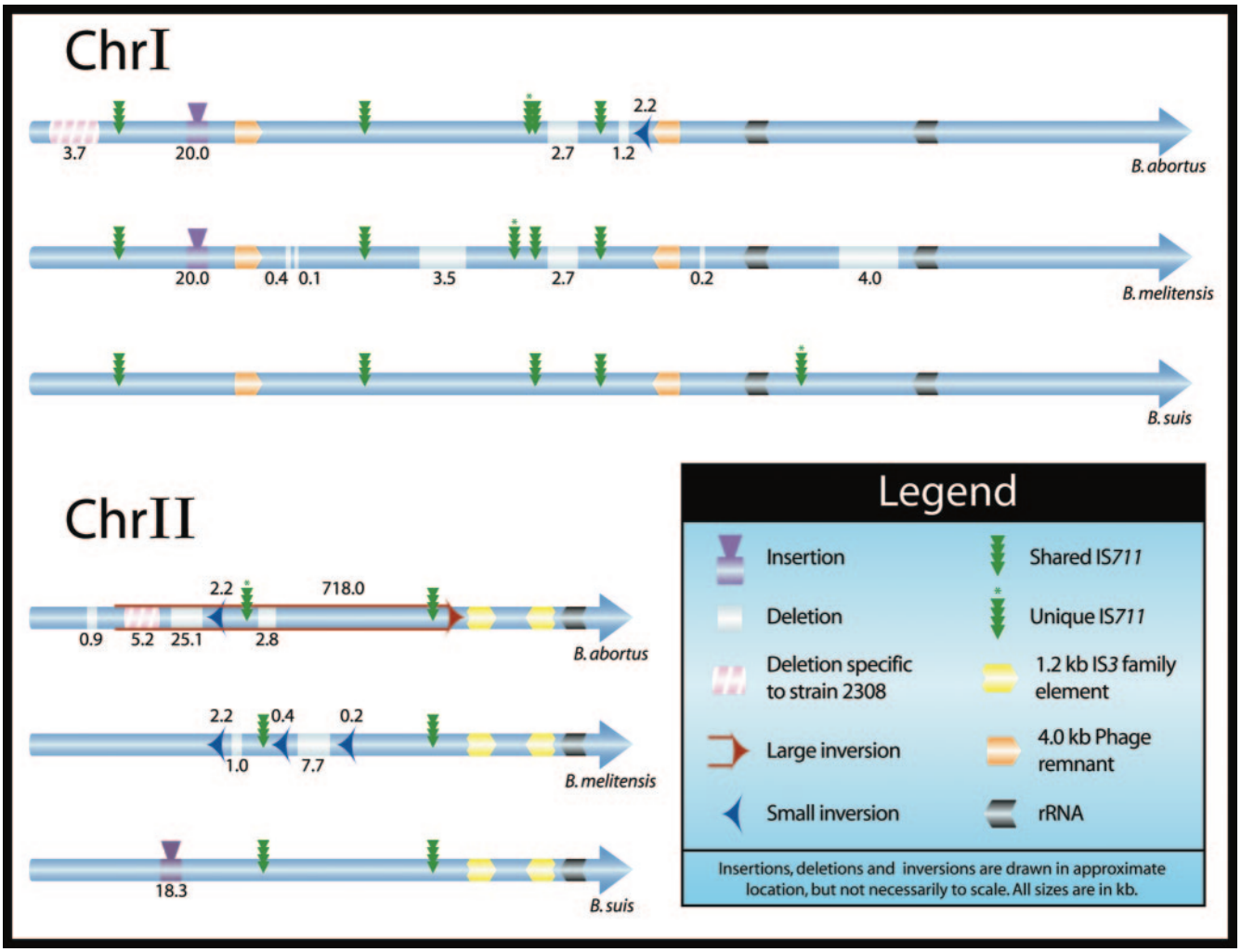

FIG. 1. Whole-genome comparison of *B. abortus* 2308, *B. melitensis* 16M, and *B. suis* 1330. A linear representation of both chromosomes displays the approximate locations and sizes (in kilobases, below features) of major insertions, deletions, and repetitive elements (including IS*711* and rRNA sequences, as well as repeated phage and IS*3* family elements). Also indicated are the approximate locations and sizes (in kilobases, above features) of inversions found within the chromosomes of the three *Brucella* spp. The hashed boxes indicate regions that were found to be missing from *B. abortus* strain 2308 but were present in strain 9-941s. Note that the large inversion in ChrII of *B. abortus* has been flipped in this diagram for better visual alignment (of the features within this inversion) with the other genomes. Refer to the inset for definitions of genome features.

performed by a shotgun method as previously described (6). All sequencing reactions were performed with BigDye Terminator v3.0 cycle sequencing chemistry and resolved with 3730xl DNA analyzers (Applied Biosystems, Foster City, CA). The whole genome sequence of *B. abortus* 2308 was obtained from 40,000 reads generating roughly 7.0-fold redundancy. Sequence finishing and polishing, assessment of final assembly quality, and gene modeling and annotation were performed as previously described (6).

Genome comparison and analysis. Whole-chromosome alignments of *B. abortus* 2308 with *B. melitensis* strain 16M and *B. suis* strain 1330 were visualized with the ACT program (which can be found at http://www.sanger.ac.uk/Software/ACT/). Due to the similarity and colinearity of the genomes, orthologs can be found by gene order and were confirmed by identifying the best reciprocal BLAST hits. Multiple alignments were performed with ClustalW. Pseudogenes were identified by comparing the *Brucella* orthologs to each other and to those of other species. In this fashion, premature stops (nonsense mutations), frameshifts, and deletion events were identified. In-frame deletion events were not included in the list of pseudogenes unless a deletion event perturbed a nonrepetitive functional domain, as identified via annotation.

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the EMBL nucleotide sequence database and assigned accession numbers AM040264 (ChrI) and AM040265 (ChrII).

RESULTS AND DISCUSSION

General features of the *B. abortus* 2308 genome. The genome of *B. abortus* strain 2308 consists of two circular chromosomes, ChrI (2,121,359 bp) and ChrII (1,156,950 bp), coding for 2,280 and 1,214 annotated open reading frames, respectively. The salient features of the genome and its comparison with the *B. suis*, *B. melitensis*, and *B. abortus* 9-941 genomes are summarized in Fig. 1 and Table 1 (as well as in Fig. S1 in the supplemental material). Although the global characteristics of the *B. abortus* 2308 genome are quite similar to the recently published sequence of the field isolate of *B. abortus* (18), a number of strain-specific deletions and polymorphisms were identified (Tables S1 and S2 in the supplemental material). The intraspecies comparison is analyzed and discussed in the context of the interspecies comparisons to two previously sequenced *Brucella* genomes (12, 35). Comparative analysis has uncovered a num-

TABLE 1. General features of *B. abortus* 2308 and comparison with *B. suis* and *B. melitensis*[a]

Property	*B. abortus* 2308		*B. suis* 1330		*B. melitensis* 16M	
	ChrI	ChrII	ChrI	ChrII	ChrI	ChrII
Size (bp)	2,121,359	1,156,950	2,107,792	1,207,381	2,117,188	1,177,787
% G + C	57.16	57.34	57.21	57.32	57.16	57.34
Total no. of ORFs[b]	2,186	1,164	2,185	1,203	2,059	1,138
No. of RNA operons	2	1	2	1	2	1
No. of tRNAs	41	14	41	14	40	14
No. of IS*711* elements	5	2	5	2	5	2
No. of pseudogenes	120	87	43	39	62	90

[a] As reported by Paulsen et al. (35) and DelVecchio et al. (12).
[b] ORFs, open reading frames.

ber of species- and strain-specific genomic features and putative physiological and metabolic differences that may help explain their host specificities and the molecular events underlying their speciation.

Comparative genomics. The genomes of the three *Brucella* species display an average of >94% identity at the nucleotide level. Despite the highly conserved genomic backbone shared by the three brucellae, several important species-specific differences have been uncovered in this work and are described and discussed below. In addition, intraspecies comparisons between *B. abortus* strains 2308 and 9-941 revealed a small number of strain-specific deletions and polymorphisms (Tables S1 and S2 in the supplemental material). Specific differences between strains 9-941 and 2308 are also discussed below in the context of cross-species comparisons.

(i) Insertions and deletions. The major insertions and deletions described in this section are illustrated in Fig. 1. Only *B. suis* possesses laterally acquired species-specific genes. Located within a single 18.3-kb cluster on ChrII, this *B. suis*-specific region encompasses 18 genes (BRA0362 to BRA0379) and appears to have been acquired recently via a phage-mediated integration event. It does not encode any obvious virulence factor but encodes many hypothetical proteins, a putative transcriptional regulator, and a type IV conjugal transfer cluster of genes previously described by Paulsen et al. (35) and is flanked by 15-bp direct repeats with a phage integrase at one end.

The only other observed integration event that differentiates between *Brucella* species (*B. suis* lacks this region) has already been described in *B. melitensis* ChrI (35) and is shared with both *B. abortus* strains 2308 (BAB1_0250 to BAB1_0279) and 9-941 (BruAb1_0245 to BruAb1_0274). This chromosomal region represents the only gene-containing region unique to *B. melitensis* and *B. abortus* and absent from *B. suis*.

In *B. abortus*, four deletion events have led to the loss of 26 complete genes and two 5′ or 3′ gene segments. The two larger deletions (25.1 and 2.8 kb) have been described in other comparative genomics analyses (18, 37), while the two smaller deletions (1.2 and 0.9 kb), also observed by DNA microarray comparative hybridization (37), encode a prokaryotic signaling, diguanylate cyclase/phosphodiesterase domain (GGDEF) protein and a ThiJ/PfpI family protease.

B. abortus strain 2308 harbors two additional deletions (5.2 kb in ChrII and 3.7 kb in ChrI) that affect an ABC periplasmic sugar-binding protein (BRA0304 in *B. suis*), a putative sugar-binding protein (BRA0302), a transcriptional regulator (BRA0301), and NarK (nitrite extrusion protein, BRA0300) in ChrII and isovaleryl-coenzyme A (CoA) dehydrogenase (BR0020), acetoacetyl-CoA synthase (BR0021), and an acyltransferase (BR0023) in ChrI. These two recent strain 2308-specific deletions appear to have occurred in regions already in a state of decay, as suggested by the many gene inactivations (see the section on gene inactivations below for more detail) found in these regions in both *B. abortus* strains and also *B. melitensis* 16M. For example, other components of the sugar ABC transport system, *narK* and *narG* (found in the same operon as *narK*) and the acetoacetyl-CoA synthase gene, all have presumed inactivating mutations in two or all three strains. Though the 3.7-kb region was found to be absent from the *B. abortus* S2308 isolate used in the DNA microarray study by Rajashekara et al. (37), the 5.2-kb deletion was not identified and may represent a very recent, isolate-specific deletion.

In *B. melitensis*, 16 complete genes, four 5′ or 3′ gene segments, and a tRNA-Glu are missing as the result of seven deletions (five in ChrI and two in ChrII) (Table S1 in the supplemental material). Interestingly, four of the five ChrI deletions in *B. melitensis* represent most of the few observed homologous recombination events within the three *Brucella* species. In one case, a transcriptional regulator (BAB1_1860) and an amino acid permease (BAB1_1861) were deleted as the result of a recombination event between two flanking and highly similar transposase-encoding genes. In another, a homologous recombination between two nearby tRNA-Glu's (tRNA-Glu1 and tRNA-Glu2 in ChrI of *B. abortus*) resulted in the loss of one tRNA and the intervening region in *B. melitensis*.

In addition, a 2.7-kb region is missing from both *B. abortus* and *B. melitensis*. This *B. suis*-specific region is located on ChrI and encompasses BR0952 to BR0954, as well as parts of the flanking genes BR0951 and BR0955 (partly deleted from the two other strains), which affects an amino acid ABC-type transporter and a glutathione *S*-transferase. We conclude that the specific deletion of this amino acid transporter, likely involved in the transport of arginine, ornithine, and lysine, is thus likely responsible for the observed inability of *B. melitensis* and *B. abortus* to oxidize these amino acids. This is the only deletion shared across *Brucella* species. Together with the above finding, the common 18.3-kb insertion and shared 2.2-kb inversion between *B. abortus* and *B. melitensis* (Fig. 1; see also the section on inversions) and the unequal distribution of pseudogenes (see the section on gene inactivations) strongly support the hypothesis that these two species arose from a common ancestor that diverged from the *B. suis* lineage (18, 29).

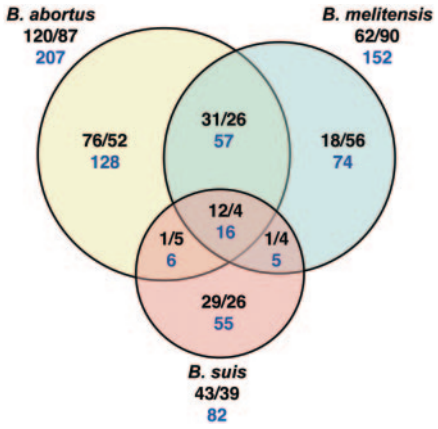

FIG. 2. Venn diagram displaying the distribution of pseudogenes among the *Brucella* genomes. The total number of pseudogenes in each section is shown in blue; distribution by chromosome is indicated (ChrI/ChrII). The total number of pseudogenes within each species genome is shown outside the circles and under the species names. Note that these numbers do not reflect genes that are absent from the genomes.

(ii) Inversions. Perhaps due to the paucity of repeated elements in the *Brucella* genomes, few rearrangements were observed among the three genomes and none of the observed inversions have occurred at repeated sequences but instead appear to be the result of nonhomologous recombinations and are likely to represent stable events. This contrasts sharply with the genomes of other pathogens with closely related neighbor species, such as members of the *Yersinia* group, in which rearrangements occur primarily at insertion sequences or other repeated sequences (7, 13).

The genome of *B. abortus* 2308 harbors the same three inversions previously described in strain 9-941 (18), including the well-characterized large ChrII inversion found in some *B. abortus* strains (29, 45), as well as a small 2.2-kb inversion shared with *B. melitensis* that disrupts the hypothetical gene BAB2_0749 and BAB2_0752, which encodes a glycoside hydrolase (Fig. S1 and S2 in the supplemental material). *B. melitensis* harbors two additional small inversions in ChrII, a 420-bp inversion within an IS*66* family element transposase (BAB2_0684 in *B. abortus*; BMEII0713/4 in *B. melitensis*) and a 204-bp inversion that disrupts a hypothetical gene (BAB2_0441 in *B. abortus*; BMEII0494 in *B. melitensis*). *B. suis* harbors no noticeable genome rearrangements compared with the genomes of *B. abortus* and *B. melitensis*.

(iii) Gene inactivations. Several small deletions in the three *Brucella* species (often intragenic) result in the generation of partial gene remnants or in the fusion of two partial genes (Table S1 in the supplemental material). A number of these events contribute to species-specific alleles, while others support the notion that *B. abortus* and *B. melitensis* are more closely related to one another. A total of 207 pseudogenes were identified in the *B. abortus* 2308 genome (versus 212 in 9-941), while 152 and 82 were identified in *B. melitensis* and *B. suis*, respectively (Fig. 2; Table S2 in the supplemental material). Very few gene inactivation differences were observed when comparing the two strains of *B. abortus*. From the total pool of inactivated genes, 16 pseudogenes are common to all three species, 57 are shared by *B. abortus* and *B. melitensis*, and only 6 and 5 are shared between *B. suis* and *B. abortus* and between *B. suis* and *B. melitensis*, respectively. It is important to note that all of these latter shared pseudogenes (between *B. suis* and either *B. melitensis* or *B. abortus*) are the result of independent mutations and do not represent commonly inherited traits. Among the 16 pseudogenes common to the three species, 9 were generated by the same event, thus strongly suggesting that they were acquired by the common ancestor of all brucellae. Likewise, most of the 56 pseudogenes shared by *B. abortus* and *B. melitensis* share the same mutation. The remainder of the pseudogenes were species specific, suggesting that a large number of independent mutations have either contributed to or occurred after their divergence from one another.

Analysis of the functional categories of the inactivated genes in brucellae revealed that one-third belong to the transport and cell envelope category, whereas transcriptional regulation and signal transduction accounted for 10% and the energy production and conversion categories accounted for 6% of the total (Fig. 3). This process of reduction of transport and cell envelope components might be the consequence of adaptation to an intracellular life-style in a protected and more stable local environment or niche that provides a constant supply of nutrients, while the dissimilar accumulation of inactivated transcriptional regulators (Fig. 3) and outer membrane structures present in brucellae might represent a contribution to, or be the consequence of, the process of adaptation to a specific animal host. This suggests that the virulence and host specificity differences observed among the three species may be the result of divergence driven by differential accumulation of pseudogenes within regulatory functions in the three species. Thus, the pattern of gene inactivation observed in brucellae would resemble that observed in the genomes of *Bordetella bronchiseptica*, *Bordetella parapertussis*, and *Bordetella pertussis*. In these species, differences in virulence and host adaptations were attributed to inactivation and loss of cell surface structural genes, transport functions, and regulatory functions (33). A similar situation has been recently observed in *Francisella tularensis* (27) and *Yersinia pestis* (7, 34), in which gene loss may contribute to virulence differences among species within the same genus. Thus, it appears that gene inactivation rather than gene deletion or gene acquisition (via horizontal transfer) may play a more important role in the speciation and host adaptation of this pathogenic group.

Metabolism. Most of the metabolic pathways that characterize prototrophic species are functional in brucellae (perhaps surprising for a well-adapted intracellular pathogen), with some interesting exceptions.

(i) Energy storage metabolites. All three *Brucella* species have lost the genes involved in the metabolism of glycogen. It is thus interesting that in the closely related alpha proteobacterial genera *Rhizobium* and *Agrobacterium*, glycogen genes are conserved and organized as a single operon (*glg*) that contains the phosphoglucose mutase (*pgm*) gene (47). The only gene of the *glg* operon conserved in *Brucella* is *pgm*, which is responsible for the synthesis of UDP-glucose, ADP-glucose, or any other sugar nucleotide derivative from them. In the genus *Brucella* as in the genera *Agrobacterium* and *Rhizobium*, *pgm* is required for effective bacterium-host interaction. *Brucella pgm*

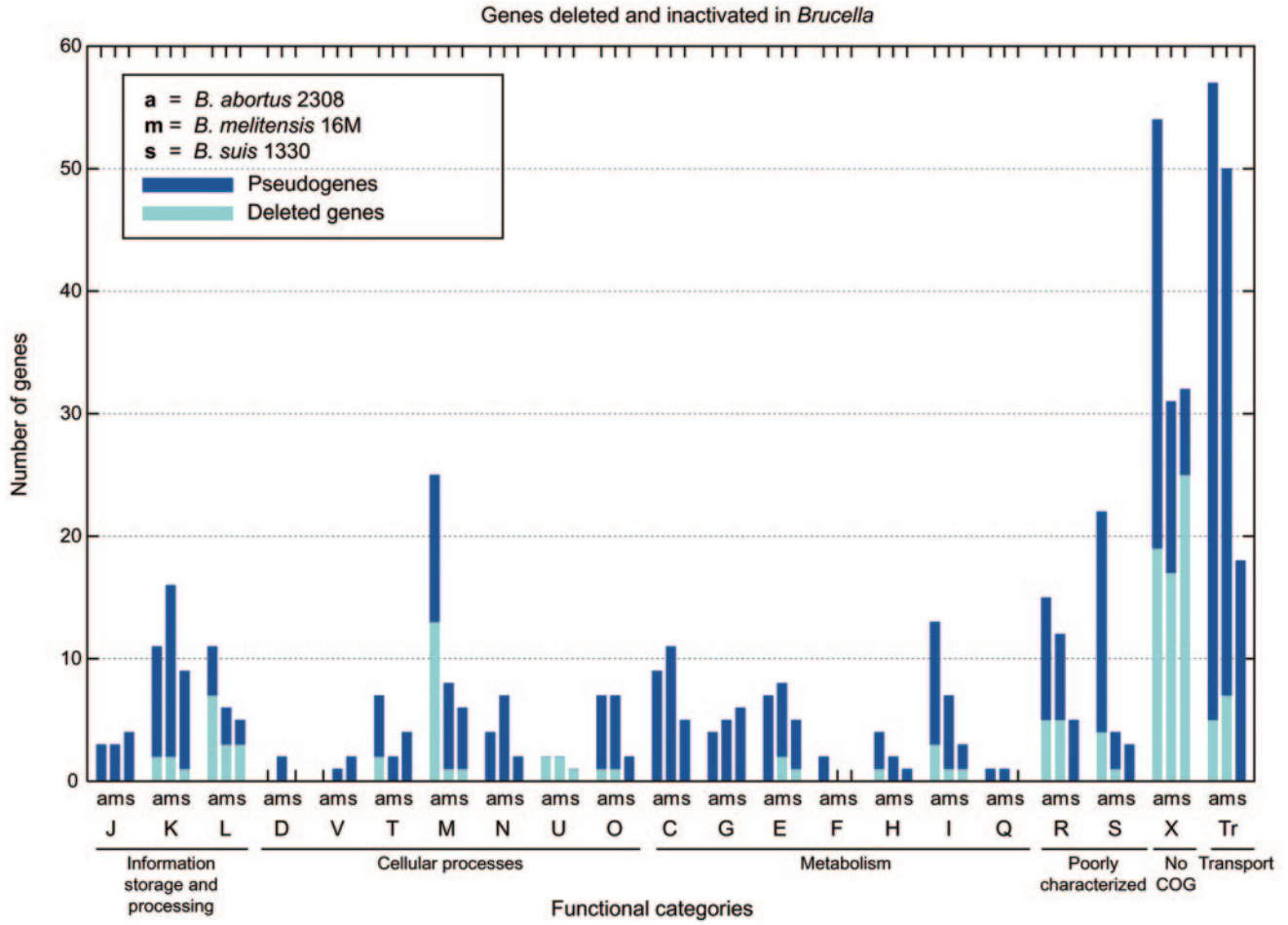

FIG. 3. Distribution of pseudogenes by functional category. Functional classifications are as follows: J, translation ribosomal structure and biogenesis; K, transcription; L, DNA replication, recombination, and repair; D, cell division; V, defense mechanisms; T, signal transduction; M, cell envelope, biogenesis, and outer membrane; N, cell motility and secretion; U, intracellular traffic, secretion, and vesicular transport; O, posttranslational modification, protein turnover, and chaperons; C, energy production and conversion; G, carbohydrate metabolism; E, amino acid metabolism; F, nucleotide metabolism; H, coenzyme metabolism; I, lipid metabolism; Q, secondary metabolite biosynthesis and catabolism; R, general function prediction only; S, function unknown; X, no cluster of orthologous groups (COG); TR, transport.

mutants contain a defective lipopolysaccharide (devoid of O antigen) and are avirulent even though they are able to replicate inside nonphagocytic cells. It is thus interesting that the only glycogen-related gene conserved in brucellae is the one required for virulence (46).

A search for the presence of other genes involved in energy storage compounds revealed that all sequenced brucellae have also lost the ability to synthesize and degrade polyhydroxybutyrate. It is likely that, as with other intracellular pathogens, brucellae have adapted to their intracellular life-style and no longer require the accumulation of energy-storing molecules.

(ii) Sugar metabolism. In most organisms, the conversion of α-D-galactose to the more metabolically useful compound glucose-1-phosphate is accomplished by the action of four enzymes that constitute the Leloir pathway. In *Brucella* species, the only enzyme present in the Leloir pathway is the UDP-galactose 4-epimerase (GalE, BAB1_0734). These findings are consistent with previous reports that GalE null mutants grow normally in minimal media with galactose (36, 39). These results suggest that the Leloir pathway is either absent or inactive and imply that, in brucellae, galactose is metabolized through an oxidative pathway likely encoded by a gene cluster located on ChrII (BAB2_0293 to BAB2_0296). Since the members of the genera *Agrobacterium* and *Rhizobium* also lack the Leloir pathway, it is likely that the absence of this pathway in brucellae represents the loss of these enzymes in an earlier ancestor of the alpha proteobacteria.

A 25.1-kb region containing 23 genes, of which most are involved in sugar metabolism and the biosynthesis of a hypothetical polysaccharide, is absent in *B. abortus* yet is present in both *B. melitensis* and *B. suis* (Table S1 in the supplemental material). Conversely, a 9-kb gene encoding Cgs, the cyclic β1-2-glucan synthetase (BAB1_0108) known to be involved in virulence (5, 8, 21), is strictly conserved in the three species. Thus, it is tempting to speculate that the set of genes absent from *B. abortus* are involved in species-specific host recognition and dispensable in this species, while the cyclic glucan synthase, a protein required for the synthesis of a virulence

factor that controls intracellular trafficking (1), must be conserved in all three species. Interestingly, Cgs is also conserved in the symbiont *Rhizobium* and is required for effective nodule colonization and symbiosis.

(iii) Nicotinamide synthesis. As mentioned above, although brucellae are relatively fastidious in terms of growth in the laboratory, they actually display a limited number of nutrient auxotrophies. All three species are auxotrophic for nicotinic acid. Sequence data confirm that the three species have an incomplete pathway for the metabolism of nicotinamide, which is metabolized through the action of PncA (EC 3.5.1.19) into nicotinic acid. Since in the brucellae the de novo synthesis of quinolinate and NAD is absent, the only precursor for the synthesis of NAD in these organisms is nicotinic acid (23). Sequence analysis confirms that all three species lack the genes for L-aspartate oxidase (EC 1.4.3.16), quinolinate synthase, and nicotinate-nucleotide pyrophosphorylase (EC 2.4.2.19), confirming the auxotrophy (17, 23) for nicotinic acid in these organisms.

(iv) Biotin synthesis. *Brucella* species are considered biotin auxotrophs (17); however, the reason for this requirement is unknown. Genome analysis reveals that the three species of *Brucella* possess all of the genes required for the synthesis of biotin (BAB2_0744 to BAB2_0748) in a highly conserved operon arrangement in ChrII. Since no hits were obtained for the *bioW*, *bioI*, *bioC*, and *bioH* orthologs, we concluded that these genes are not present in the *Brucella* genome. However, the genes *bioW*, *bioI*, and *bioC* are known to be dispensable and their absence should not affect their ability to synthesize biotin (15). Furthermore, the last gene of the *B. abortus bio* operon (BAB2_0748) displays high homology to *bioZ*, a fatty acid synthase gene which, in *Mesorhizobium loti*, is involved in the synthesis of pimeloyl-CoA. Thus, in brucellae, this gene could compensate for the absence of the pimeloyl-CoA synthase gene *bioH* (43). Our genome comparisons failed to uncover the reason for biotin auxotrophy in the brucellae.

Interesting are the high similarities of the *Brucella* and *M. loti bio* operons and the fact that the *M. loti bio* operon is located within its symbiotic island, a region that can be lost or horizontally acquired (43). The possibility that the *Brucella bio* operon was horizontally acquired is supported by a drastic change in G+C content (32 to 45% compared with 57.3% for ChrII) observed at both ends flanking the *bio* operon (ChrII, bp 739700 to 739900 and bp 744920 to 745140).

(v) PC biosynthesis. One of the outstanding characteristics of the *Brucella* cell envelope is the presence of phosphatidylcholine (PC) as one of the major membrane-forming phospholipids (32), a feature characteristic of eukaryotic membranes. Though not present in most prokaryotes, PC has also been found in a diverse array of bacteria, including photosynthetic bacteria, symbionts, and pathogenic bacteria capable of causing persistent infections, such as *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Borrelia burgdorferi* (41). The genomes of brucellae encode orthologs of the enzymes responsible for methylating phosphatidylethanolamine and for directly condensing choline with CDP-diacylglycerol into PC, phosphatidyl-*N*-methylethanolamine *N*-methyltransferase (Pmta), and PC synthase (Pcs), respectively. The Pmta coding sequence is located on ChrI in all three species (BMEI2000, BR2127, and BAB1_2131). However, a closer inspection of the sequences indicates different amino acid substitutions spanning the conserved consensus motif (VLELGXGXG) in the *S*-adenosylmethionine-utilizing methyltransferases in all three species. This suggests that the methylation pathway may not be functional in this genus.

The Pcs coding sequence is located on ChrII of the three species (BMEII0695, BRA0572, and BAB2_0668). The Pcs consensus motif characteristic of CDP-alcohol phosphatidyltransferases ($DGX_2ARX_{12}GX_3DX_3D$) is absolutely conserved, suggesting that this gene could be the one responsible for PC synthesis in the genus. This is supported by experimental data which indicate that the brucellae are unable to synthesize PC de novo with phosphatidylethanolamine as a precursor, suggesting that the pathway for PC formation is condensation of choline provided by the host with CDP-diacylglycerol (D. J. Comerci, unpublished results).

(vi) Electron transfer. Genome analysis revealed that the respiratory chain of brucellae consists of a branched pathway. Electrons may be transferred from the quinone pool to the terminal oxidases through either the cytochrome bc_1 complex or the alternative route with quinol oxidase. Genes BAB1_1557 to BAB1_1559 encode the Rieske Fe-S protein and cytochromes *b* and *c* of the bc_1 complex. This complex transfers electrons from the oxidized quinol pool to the type *c* cytochromes, which subsequently transfer them to the terminal cytochrome *c* oxidase. Indeed, the terminal branch for the bc_1 complex is represented by the cytochrome *c* oxidase complexes aa_3 (BAB1_0492 to BAB1_0497) and cbb_3 (BAB1_0385 to BAB1_0392), but only the latter seems to be functional since the cytochrome *c* oxidase assembly protein CtaG is inactivated by a frameshift (BAB1_0495 to BAB1_0496). The cbb_3-type complex is encoded by the *fixNOQP* operon present in several rhizobia, species in which this is required for microaerobic respiration during root nodule symbiosis. Experimental data indicate that the cytochrome bc_1 pathway is dispensable for growth under vegetative or intracellular conditions, suggesting that there is an alternate route for respiration (24).

Examination of the *B. abortus* genome indicates that it harbors genes encoding the terminal quinol oxidase bo_3 complex (BAB1_0038 to BAB1_0042) and the *bd* complex (BAB2_0727 to BAB2_0730). However, as in the previous example, a critical gene in the pathway is missing. In this case, the *qoxB* gene encoding subunit II of the bo_3 complex is inactivated by a frameshift (BAB1_0039 to BAB1_0040), suggesting that only the cytochrome–D-ubiquinol oxidase *bd* complex is functional. Previous experimental data have revealed that the *bd* complex is essential for intracellular survival and virulence in *B. abortus* 2308, further suggesting that this is the main electron transfer route (14) in the brucellae. In other bacteria, the cytochrome *bd* complex has a high affinity for oxygen (44) and is the main cytochrome expressed under microaerobic conditions in *Escherichia coli* and in nitrogen-fixing bacteria. It can thus be speculated that the presence of cytochromes with a high oxygen affinity may represent an important adaptation of brucellae to their intracellular life-style.

Potential virulence modulators. Brucellae do not possess classic virulence factors; rather, those already identified play roles facilitating cell invasion and survival by subverting the innate cellular defense mechanisms (1, 10, 42). Genetic ele-

ments and cellular systems that fall into this category include the following.

(i) Type IV secretion system. In pathogenic bacteria, secretion systems are usually involved in pathogen-host interactions by delivering signals (including toxins) into host eukaryotic cells. The three species of *Brucella* have a complete and functional type IV secretion system, which is highly conserved at the nucleotide level, suggesting high selective pressure for its conservation (4). This key virulence determinant is 99% conserved at the DNA level among the three species, a feature that contrasts with the high variation observed in the putative type III secretion system and flagellar export system region described below.

(ii) Flagella. Brucellae are nonmotile bacteria; however, their genomes contain a large complement of class II and class III flagellar genes (12, 28). The organization of these genes is the same in the three species, with 44 genes arranged in three loci on ChrII. Another two genes (a second copy of *motB* and the gene for the flagellum-specific muramidase, *flgJ*) are located in different regions of ChrI. Closer examination revealed that of the 44 genes, 7 are pseudogenes in *B. melitensis*, compared with 4 each in *B. suis* and in *B. abortus* (Table S3 in the supplemental material). Only *fliG* (encoding the flagellar motor switch) is a pseudogene in all three *Brucella* species, while the distribution of the other flagellar pseudogenes varies across the species. The inactive genes contain either single point mutations or small deletions and are essential for the activity (*fliG*, *fliM*, and *motC*) or the assembly (*fliI*, *flgA*, and *flhA*) of the flagellum or are key structural proteins of this organelle (*flgF* and *flgI*) (3). Thus, the lack of motility in brucellae is a reflection of inactivation of key flagellar genes and the absence of chemotactic systems.

Recently, however, it has been shown that under specific growth conditions, *B. melitensis* is able to build up a sheathed flagellum-like structure that seems to be necessary for establishing a chronic infection process in mice by an as-yet-unknown mechanism (16). Besides the putative role that the flagellum could play in virulence, there is no evidence to date for *Brucella* motility. Our comparative analysis of the flagellar clusters in brucellae reveals that these species possess the potential to generate structures that are derived from the flagellum but that are not related to motility since most of the genes encoding basal-body or motor components have been inactivated. These putative functional flagellum-related genes could thus be responsible for the flagellum-like structure reported by Fretin et al. (16). In summary, the differential distribution of flagellar pseudogenes across the three species, plus the potential expression of different flagellum-related components in the brucellae, could play a role in host specificity and the virulence process in these organisms.

(iii) Regulatory elements. Bacterial alternative RNA polymerase sigma factors are key global adaptive response regulators that have been shown to regulate virulence mechanisms in several bacterial pathogens, including *Salmonella enterica* serovar Typhimurium (20), *L. pneumophila* (2), and *Mycobacterium tuberculosis* (9). In particular, extracytoplasmic function (ECF) sigma factors, which mainly control cell envelope synthesis, secretory functions, and periplasmic proteins, may play an important role in *Brucella* pathogenesis.

All three species of *Brucella* carry a highly conserved ECF gene in ChrI, while a 211-amino-acid paralog encoded in ChrII is present only in *B. abortus* and *B. suis*. In *B. melitensis*, a GGGG insertion in the 5′ region of the ECF gene has generated a fusion with a downstream hypothetical gene, which now putatively encodes a fusion protein of 413 amino acids. Though the significance of this difference is not known, it is interesting that other species of the alpha proteobacteria phylogenetically close to the brucellae have a variable number of ECFs. The free-living species *Caulobacter crescentus* and *Agrobacterium tumefaciens* have 13 and 11 genes, respectively. In contrast, *Brucella* spp. and the facultative endosymbionts *M. loti* and *Sinorhizobium meliloti* have only two ECF paralogs, while the strict intracellular pathogens such as *Rickettsia* spp. lack ECF sigma factors entirely. This correlation suggests that in the alpha proteobacteria, these sigma factors do not confer any selective advantage as the organisms evolve toward a more host-dependent life-style.

(iv) Proteins containing autotransporter domains. Autotransporters are members of a large family of exported proteins that encode an integral outer membrane pore that enables them to cross the outer membrane (19). Members of this family have been implicated as important virulence factors in many gram-negative pathogens. The three *Brucella* genomes contain four genes with putative autotransporter β domains at the C terminus (Table S4 in the supplemental material); however, these genes encode four different proteins since they show low or no sequence similarity outside of this domain. The four autotransporters contain a number of hemagglutinin (Pfam PF05662) and heptapeptide (Pfam PF05658) motifs that are present in bacterial adhesins and invasins. Based on the analysis of the gene sequences, it is clear that many of them are likely inactive (Table S4 in the supplemental material). We have found that all four autotransporters in *B. melitensis* are pseudogenes, each with a variety of different inactivating mutations. As a consequence of a frameshift, *B. suis* BR0072 lost the signal peptide sequence and is thus predicted to start a few bases downstream, at an alternative ATG codon, giving an almost complete coding region lacking the signal peptide sequence that is present in *B. abortus* (BAB1_0069). Thus, though BR0072 seems likely to be a pseudogene in *B. suis*, it remains to be seen if it is functional or able to be secreted into the periplasm via a Sec-independent mechanism. The gene BAB1_0069 also differs from its *B. suis* (BR0072) and *B. melitensis* (BMEI1873/4) orthologs in the number of internal repeated tandem sequences, which contain hemagglutinin motifs and are commonly found in autotransporter genes. Similar differences in repeated motifs were observed in the *B. abortus* BAB2_0167/BAB2_0168 autotransporter, which is much larger than its *B. suis* (BRA0173) and *B. melitensis* (BMEII1069/70, a pseudogene) counterparts but carries a number of mutations which likely render it inactive. A third autotransporter appears to be functional in *B. abortus* (BAB2_1107) and *B. suis* (BRA1148) but a pseudogene in *B. melitensis* (BMEII0148/9). As previously described, the fourth autotransporter carries inactivating nonsense mutations in *B. abortus* (BAB1_2013 and BAB1_2014) and *B. melitensis* (BMEI0058) but is full length in *B. suis* (BR2013). Since almost all of the autotransporters known are involved in functions related to the invasion process, either by direct interaction with the host or with the

extracellular matrix, it is tempting to speculate that the difference in the number of active autotransporters, and the variation within them, may play a role in the ability of each species to interact with its host and may thus be an important contributor to the host specificity displayed by this group.

Concluding remarks. Twenty years ago, DNA-DNA hybridization studies revealed the high degree of homology shared by the six recognized *Brucella* species. This led to the proposition that *Brucella* constitutes a monospecific genus and that *B. melitensis* be used as the exclusive species, while the others should be considered only biovars of *B. melitensis* (49). However, this single-species organization does not reflect accurately the observed differences in pathogenicity, host preference, and evolutionary history, and by and large, the classical scheme of six species is still preferred. The genomic comparisons presented in this work support the notion that the three main *Brucella* species have a singular evolutionary history and that species-specific DNA sequences and pseudogene distributions might correlate with different host preferences.

Recently, it has been hypothesized that *B. melitensis* and *B. abortus* shared a common ancestor and became isolated about 20 million years ago, when radiation of artiodactyls occurred (30). The genomic rearrangements, species-specific DNA sequences, and distinct patterns of gene inactivation independently suggest that *B. abortus* and *B. melitensis* share the same lineage, which differs from the *B. suis* lineage, which has undergone fewer genetic mutations since it diverged from the most recent common ancestor of all brucellae. Indeed, both *B. melitensis* and *B. abortus* seem to be more restricted in host range (can cause abortion in sheep, goats, cattle, and all members of the clade *Ruminantiae*), whereas *B. suis* can infect a broader range of animals (swine, reindeer, rabbits, and dogs) and is the most diverse in genomic structure and host preference (31).

This evolutionary history is highly reminiscent of the radiation of *Cetartiodactyla* during the Paleocene-Eocene period (between 50 and 20 million years ago), where the family *Suidae* (pigs) is the deeper clade and the suborder *Ruminantiae*, which includes both the subfamilies *Bovinae* (cows and buffaloes) and *Caprinae* (goats and sheep), evolved later. In this regard, the completion of the genome sequences of the marine brucellae (from both pinnipeds and cetaceans) and of *Brucella ovis* would provide valuable information necessary to complete this hypothesis.

The genomic analyses in this work show that the majority of the identified virulence genes were conserved among the three species and thus that differences in pathogenicity could not be attributed to variations in gene complement but rather may be the consequence of dissimilar gene inactivation of cell surface components and of transcriptional regulators. It is tempting to speculate that species-specific gene inactivations, such as in outer membrane proteins, autotransporters, ABC transporters, and regulatory genes, may account for cell surface variations and may be involved in environmental adaptation and nutrient scavenging. Interestingly, the most frequent number of species variations, including pseudogenes, occurs precisely within these functional categories. Accordingly, whereas the 11.7-kb *virB* operon encoding the important type IV secretion system is highly conserved in all four genomes, high variability and differential gene inactivations characterize what appears to be a remnant flagellar type III secretion system implicated in the production of a proteinaceous appendage with a role in the chronic form of brucellosis infections. Such dichotomy reaffirms the nature of the *virB* operon as an essential component of the virulence process in members of this group, while the high variation and heterogeneity observed in the flagellar loci is more consistent with a putative role in host specificity.

Finally, numerous losses affecting general metabolic processes in these organisms, such as the elimination of a gene cluster involved in sugar nucleotide synthesis, sugar modification, and polysaccharide synthesis, as well as the loss of several other pathways, such as the synthesis of the energy and carbon storage compounds glycogen and polyhydroxybutyrate, the vitamin biotin, and choline, plus the apparent selection for a functional high-affinity respiratory mechanism, are consistent with adaptation of brucellae to the protected, nutrient-poor, low oxygen tension environment of its intracellular niche.

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